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Different Removal of Ultraviolet Photoproducts in Genetically Related Xeroderma Pigmentosum and Trichothiodystrophy Diseases[1]

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ABSTRACT

To understand the heterogeneity in genetic predisposition to skin cancer in different nucleotide excision repair-deficient human syndromes, we studied repair of cyclobutane pyrimidine dimers (CPDs) and of pyrimidine(6-4)pyrimidone (6-4PP) photoproducts in cells from trichothiodystrophy (TTD) patients. TTD is not associated with increased incidence of skin cancer, although 50% of the patients are photosensitive and carry a defect in the nucleotide excision repair pathway, similar to Xeroderma pigmentosum patients. However, in striking contrast to TTD, Xeroderma pigmentosum is highly prone to cancer. To address this apparent paradox, two types of studies were conducted: (*a*) reactivation of UV-irradiated plasmids harboring actively transcribed reporter genes, with or without photolyase treatment before transfection of SV40-transformed fibroblasts; and (*b*) the kinetics of removal of UV-induced CPDs and 6-4PPs in genomic DNA by immunoblot analysis using lesion-specific mAbs in SV40-transformed and untransformed fibroblasts representative of all genetic TTD complementation groups. Results showed that all cell lines from photosensitive TTD patients efficiently express *Cat* or *luciferase* genes in transfected plasmids carrying non-CPD lesions, including 6-4PP, and display wild-type or near-wild-type (50–70% in 3 cell lines) 6-4PP repair in the overall genome after immunoblot analysis. However, CPD lesions (the repair of which is defective in the overall genome) also block the expression of the reporter gene in transfected plasmids. Two cell lines from nonphotosensitive TTD patients showed wild-type levels of repair for both photoproducts in overall genome. A model on the lesion-specific repair in the context of the molecular defect in TTD is proposed. The implication of the defective CPD repair and efficient 6-4PP repair subpathways in cancer prevention in TTD patients is discussed.

INTRODUCTION

TTD,[3] XP, and CS share defective NER abnormal cellular responses to DNA damage but have strikingly different pathological manifestations, notably with regard to skin cancer predisposition. The study of these diseases may, therefore, provide important clues to understand environmental carcinogenesis. TTD is a rare autosomal recessive disease characterized by brittle hair with reduced sulfur content, impaired mental and physical development, a peculiar face, and ichthyosis (1). Photosensitivity has been reported in approximately 50% of the cases and is associated with an increased cellular UV sensitivity (2). This cellular phenotype is similar to that observed for XP cells with defective NER (3). Major clinical manifestations in all XP patients are photosensitivity, sun-induced skin pigmentation abnormalities, and, in some individuals, markedly impaired physical development and accelerated neurological degeneration (4). The third defective NER-related disease, CS, is characterized by markedly growth and mental retardation, neurological degeneration due to intracranial dysmyelination, and different extents of sun sensitivity (5). A subset of photosensitive CS patients also show typical XP clinical symptoms and a cellular DNA repair-deficient phenotype (XP/CS patients). The major clinical consequence of solar sensitivity in almost all XP patients is a 1000-fold increased frequency of skin cancer. However, cutaneous cancers have never been reported in CS and TTD, even in most severely affected individuals (6, 7).

Complementation analysis by somatic cell fusion or microinjection of DNA repair genes revealed that NER-defective XP patients belong to 7 genetic groups (XP-A to XP-G), classical CS patients belong to 2 groups (CS-A and CS-B), a third class of combined XP/CS patients fall into 3 XP groups (XP-B, XP-D, and XP-G), and photosensitive TTD patients belong to 3 groups [TTD-A (a new NER group), TTD/XP-B, and TTD/XP-D] because cells from latter patients fall into the XP-B and XP-D groups, respectively (3, 8–12). In terms of cellular DNA repair properties, XP, CS, and TTD patients present heterogeneous responses to UV damage: reduced UDS in XP and TTD cells and defective repair in the actively transcribed genes with normal levels of UDS in CS cells (13).

Numerous studies prove the direct involvement of defective NER in neoplasic development in XP. Consequently, to understand the molecular basis for the remarkable differences of skin cancer proneness in these three diseases, it is important to resolve the role of unrepaired UV-induced DNA photoproducts (14). The predominant types of UV-induced lesions are CPD and 6-4PP dimers, both of which are removed by the NER pathway. In most XP cells, including the XP-A and XP-D groups, repair of both CPD and 6-4PP is defective (15, 16). In CS cells, representative of photosensitive cancer-free patients, it has been shown that CPD, but not other lesions, block the expression of *cat* gene pRSVcat vector (17). Finally, some TTD cells from photosensitive patients (without skin cancers) have been described with a reduced rate of repair of 6-4PP (18). In this context, it was of primary interest to further investigate the repair properties of these two major lesions in TTD cells, representative of cancer-free patients with or without photosensitivity, and to compare their repair properties to XP and CS cells in our experimental conditions.

The specific repair of these two lesions can be followed by using different experimental procedures: (*a*) incision of the damaged DNA

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[3] The abbreviations used are: TTD, trichothiodystrophy; XP, Xeroderma pigmentosum; CS, Cockayne's syndrom; NER, nucleotide excision repair; UDS, unscheduled DNA synthesis; CPD, cyclobutane pyrimidine; 6-4PP, pyrimidine(6-4)pyrimidone; nfm, no-fat milk.

by purified uvrABC with or without prior enzymatic reversion of CPD with photoreactivating enzyme (19, 20); (*b*) detection of UV-induced lesion by HPLC analysis after enzymatic and chemical cleavage of genomic DNA (21); (*c*) immunological analysis of genomic lesions with damage-specific antibodies (22, 23); or (*d*) ability of cell extracts to perform repair synthesis *in vitro* (24). In this work we used two approaches: (*a*) the study of reactivation of UV-irradiated plasmids harboring reporter genes, with or without photolyase pre-treatment, transfected into SV40-transformed fibroblasts; and (*b*) repair studies of both lesions in genomic DNA from primary and SV40-transformed fibroblasts by immunoblot analysis using specific mAbs (25–27). We found that all TTD cells show wild-type or near-wild-type levels of 6-4PP repair in a transcribed plasmid as well as in the overall genome, whereas CPD repair was significantly reduced in photosensitive patients, and normal repair of both CPD and 6-4PP was found in the overall genome in cells of nonphotosensitive individuals.

MATERIALS AND METHODS

Cell Culture Conditions. All cell lines used are listed in Table 1. Fibroblasts amplified from skin biopsies were grown in modified Eagle's medium supplemented with 15% FCS and 1% antibiotics (penicillin, streptomycin, and fungizone). Cells were split 1:2 or 1:3 every 1 or 2 weeks, according to the growth rate of each cell line. SV40-transformed fibroblasts were established as described previously (28) and were grown in the same medium and split 1:10 every week.

DNA Treatments and Determination of UV Photoproducts. The plasmid DNA preparation was carried out by standard alkaline lysis procedure and a double CsCl density gradient. PRSVcat (29) and pRSV luciferase (pRSVL) (30) plasmids were irradiated with a germicidal UV lamp (General Electric) at a fluency rate of 4 J/m^2/s for the indicated UV doses. Three hundred-ng aliquots were incubated for 30 min at 30°C in the presence or absence of 3 units of *Anacystis nidulans* photolyase (31) under a UVA illumination (lamp Uvastar-KG1) with a fluency of 30 J/m^2/s. DNA samples were then incubated with T4 endonuclease V (32) in 10 mM Tris-HCl (pH 8.0)-100 mM NaCl-1 mM EDTA at 37°C for 30 min. Samples were subsequently electrophoresed through 0.8% agarose gels. The conversion of supercoiled form I into relaxed circular form II was quantified by densitometric scanning of negative photographic pictures of gels. Assuming a Poisson distribution for UV photoproducts and that an average number of one T4 endonuclease-sensitive site converts 37% of form I molecules into form II, the D_{37} was calculated to be 20 J/m^2 for one CPD in pRSVcat plasmid (data not shown). This is similar to values reported previously under comparable experimental conditions (33, 34). The ratio of 6-4PP over CPD was measured using an immuno slot-blot assay on plasmids before photolyase treatment with the help of mAbs TDM-2 and 64M-2. The specificity of these antibodies have been documented already, showing no detectable cross-reactivity between CPD and 6-4PP, respectively, for T-T, T-C, C-T, or C-C (25). Two others mAbs, H3 and 1F7, reacting against CPD and 6-4PP, respectively, were obtained and characterized as described previously (26, 27). Fig. 1*A* shows that photolyase treatment completely eliminated the binding of TDM-2 antibodies but had only a minor effect on the binding of 6-4 M-2 (Fig. 1*A*, compare *top* to *bottom*). This confirms the antibody specificity for CPD and 6-4PP photoproducts and monitors the complete monomerization of CPD by the photolyase treatment. The densitometric scanning of the blot in Fig. 1*A* shows linearly increasing binding of both 64M-2 and TDM-2 antibodies as a function of the UV dose. The ratio of 6-4PP/CPD binding was calculated to be equal to 18.1%. This value, which is identical to that found after HPLC analysis (21), was obtained assuming that all DNA photoproducts are detected by antibodies irrespectively of their affinity to DNA photoproducts in saturation condition (Fig. 1*B*).

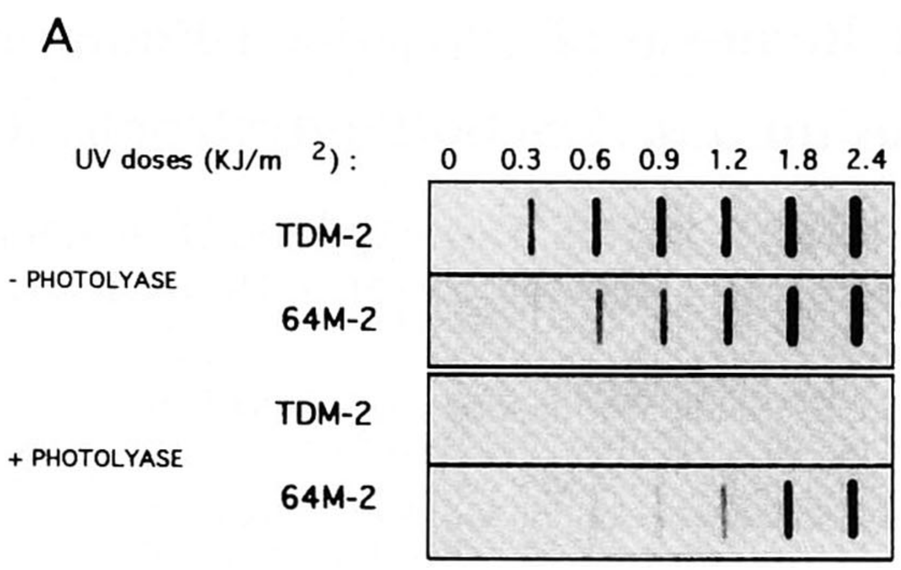

Fig. 1. Rates of formation of CPD and 6-4PP in UV irradiated plasmid by immuno slot-blot assay with mAbs. *A*, autoradiogram of slot-blots containing pRSVL plasmid DNA irradiated at indicated doses with or without photolyase treatment and probed with anti-CPD (*TDM-2*) or anti-6-4PP (*64M-2*) antibodies. Times of exposition of autoradiograms for TDM-2-probed blots of DNA samples without or with photolyase treatment were 2 and 8 min, respectively. *B*, densitometric quantification of autoradiograms in *A* for DNA samples probed with TDM-2 (⊡) and 64M-2 (♦) antibodies without photolyase treatment. *Points*, mean for duplicate samples in two different experiments; *bars*, SEM.

DNA Transfections and Cat and Luciferase Assays. These procedures for DNA transfection and the cat and luciferase assays have been described previously in detail (35).

Determination of CPD and 6-4PP Photoproducts in Genomic DNA by Immuno Slot-Blot Analysis. Exponentially growing fibroblasts were UV irradiated at 254 nm at a fluency of 0.2 J/m^2/s with 15 J/m^2. At indicated times after irradiation, fibroblasts were scraped from the dishes, and cell pellets were stored at −20°C until processing. Fibroblasts were thawed at room tempera-

Table 1 *Cell lines used in this study*

Phenotype	Genetic group	Cell line: Untransformed	Cell line: SV40-transformed	Source
Wild-type		MRC5	MRC5V1SV	A. R. Lehmann
		C5RO	C5ROLas	J. H. J. Hoeijmakers
XP	XP-A	XP4LO		C. Arlett
			XP12ROSV	D. Bootsma
	XP-A revertant		XP129	J. E. Cleaver
	XP-D	XP16PV		M. Stefanini
			XP6BESV	ATCC[a]
Photosensitive TTD	TTD/XPD	TTD1VI	TTD1VILas	A. Sarasin
		TTD2VI	TTD2VILas	A. Sarasin
		TTD3VI	TTD3VILas	A. Sarasin
		TTD8PV		M. Stefanini
		TTD9VI	TTD9VILas	A. Sarasin
		TTD1RO	TTD1ROLas	W. J. Kleijer
	TTD-A	TTD1BR3	TTD1BRNeo	A. R. Lehmann
	TTD/XPB	TTD6VI	TTD6VILas	A. Sarasin
Nonphotosensitive TTD		TTD5VI		A. Sarasin
		TTD8VI		A. Sarasin
CS	CS-A		CS3BESV	A. R. Lehmann

[a] ATCC, American Type Culture Collection.

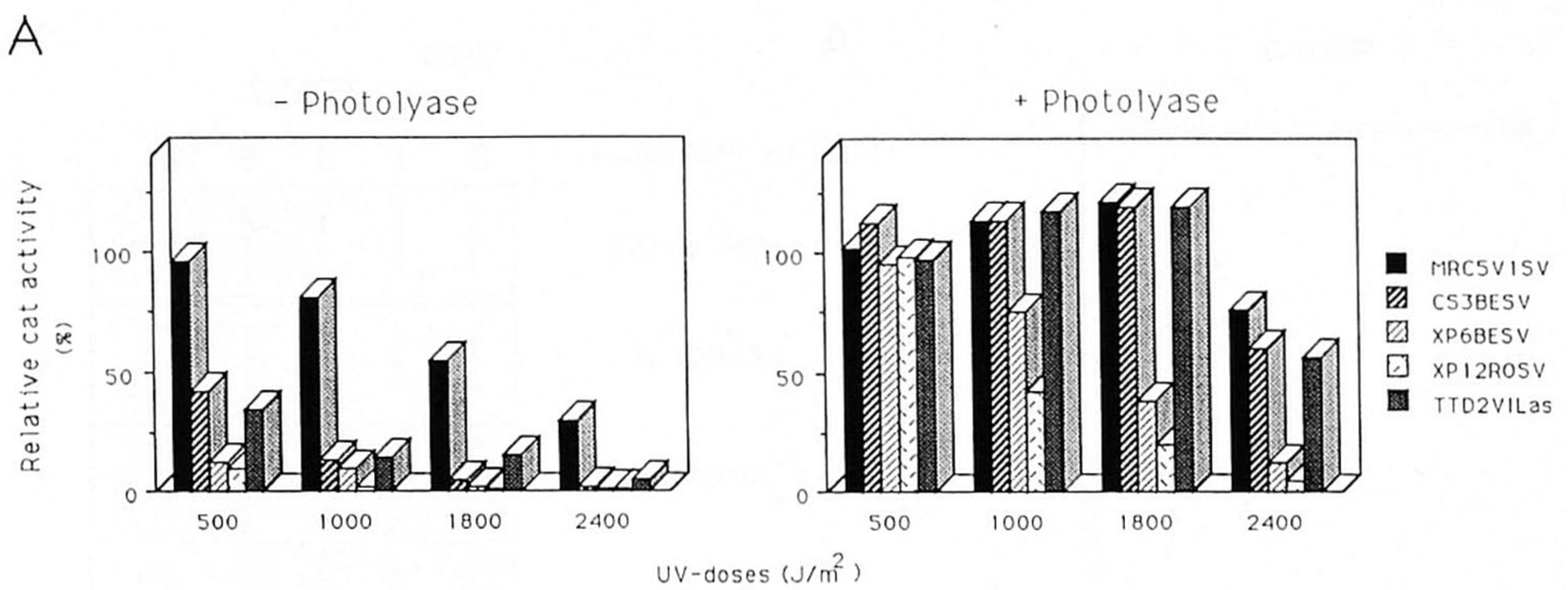

Fig. 2. Reactivation levels of UV-irradiated pRSVCat (*A*) or pRSVL (*B*) plasmids without or with photolyase treatment. Plasmids were irradiated at indicated doses and treated or not with photolyase before transfections, as indicated in "Materials and Methods." Relative cat or luciferase activities were expressed as the ratio of the enzyme levels detected in cells transfected with irradiated over unirradiated plasmid. *Columns*, mean of three separate experiments. SE for each experiment, about ± 20%.

ture; 0.5 ml of lysis buffer containing 10 mM Tris-HCl (pH 8.0), 0.2% SDS, 0.1 M NaCl, and 2 mM EDTA was added; and fibroblasts were left to lyse for 30 min. Samples were first incubated with 10 μg/ml of RNase for 4–5 h, and then with 20 μg/ml of proteinase K at 37°C overnight. DNA samples were extracted with phenol and 24:1 chloroform/isoamylic alcohol treatments, followed by ethanol precipitation. For immuno slot-blot analysis, 0.5 μg of purified DNA was loaded on nitrocellulose membrane (BA 83-S, Schleicher & Schuell). Membranes were saturated with PBS containing 5% of lyophilized nfm for 1 h at 37°C (or 8 hours at 4°C) and then incubated with TDM-2 or 64M-2 antibodies (diluted 1:500 in 0.5% nfm PBS) for 1 h at 37°C. After extensive washing with the above buffer, blots were incubated with a 1:2000 dilution of a second antimouse horseradish peroxidase-conjugated antibody (Calbag, San Francisco, CA) for 30 min at room temperature. Blots were then extensively washed with 0.5% nfm-PBS and PBS alone before being processed with enhanced chemiluminescence (Amersham) solutions and exposed to X-ray films. For precise quantification of CPD and 6-4PP, films from different exposure times were scanned to calculate absorbance in the linear range of the film.

RESULTS

Expression of *cat* and *luciferase* Reporter Genes in UV-irradiated pRSVcat and pRSVL Plasmids with or without Photolyase Treatment. SV40-transformed fibroblasts have been used for DNA transfections because of their higher transfection efficiency and expression of foreign genes compared to primary fibroblasts. TTD2VILas cells, belonging to the TTD/XPD group, were analyzed and compared to several other cell lines used as different controls: wild-type (MRC5V1SV), CS-A (CS3BESV), XP-A (XP12ROSV), and XP-D (XP6BESV). The two latter cell lines have deficient repair of both CPD and 6-4PP lesions (16, 21), whereas CS-A cells (CS3BESV) are able to express a UV-damaged plasmid-carried reporter gene after photolyase removal of CPD (17). Fig. 2*A* shows that, when these cell lines are transfected with UV-irradiated pRSVcat plasmid without previous photolyase treatment, there was a dose-dependent inhibition of cat expression, but at different rates, for all cell lines. MRC5V1SV cells present a cat activity decreasing from 96 to 30% at increasing doses from 500 to 2400 J/m^2. Lower activity was found in CS3BESV (42 to 2%) and TTD2VILas (34 to 5%) cells. An even lower activity was detected in XP12ROSV (10 to 1%) and in XP6BESV (12 to 1%) cells. These results indicate that wild-type MRC5V1SV are able to reactivate UV-irradiated pRSVcat plasmid, whereas CS3BESV and TTD2VILas cells are defective in such a reactivation but less defective than a XP-D and a XP-A cell strain. In the presence of photolyase treatment (Fig. 2*A*, *right*), wild-type cells recovered 100% of cat activity at all UV doses, except for 2400 J/m^2, where the activity was 76%. A similar extent of recovery of cat activity was found for CS3BESV and TTD2VIlas cells for all UV doses, whereas significantly reduced cat activity was still measured for both XP12ROSV and XP6BESV cells for doses equal to or higher than 1000 J/m^2. Therefore, 6-4PP and other nondimer photoproducts are not transcription-blocking lesions in TTD2VILas cells or in CS or wild-type cells.

We extended this analysis to four other TTD cell lines (TTD1VILas, TTD1ROLas, TTD6VILas, and TTD1BRneo) by using a second reporter pRSVL plasmid harboring the *luciferase* gene (Fig. 2*B*). This gene presents major advantages, such as more rapid processing of samples and enzymatic assay (35). In these experiments, a second wild-type C5ROLas line was included as control cell, and CS3BESV cells were included as positive control (not shown in Fig. 2*B*). The other TTD lines cover all genetic groups for TTD: two more TTD/XP-D lines (TTD1VILas and TTD1ROLas), and two cells representative of two other TTD groups [TTD-A (TTD1BRneo) and TTD/XP-B (TTD6VILas). Results showed that, in the absence of photolyase treatment (Fig. 2*B*, *left*), dose-response inactivation of luciferase expression is observed in all cell lines, similar to that observed with pRSVcat plasmid (Fig. 2*A*). Furthermore, in these four other TTD lines, the relative enzymatic activities were very similar to each other and to TTD2VILas cells (Fig. 2*A*). This indicates that transcription-blocking UV lesions remain in plasmids transfected in all these TTD cells. In the presence of photolyase treatment, all TTD cells show recovery of cat or luciferase activity similar or identical to wild-type MRC5V1 and C5ROLas or to CS3BESV cells. This indicates that wild-type reactivation of UV-irradiated pRSV plasmids after *in vitro* removal of CPD is a common property to all TTD cells.

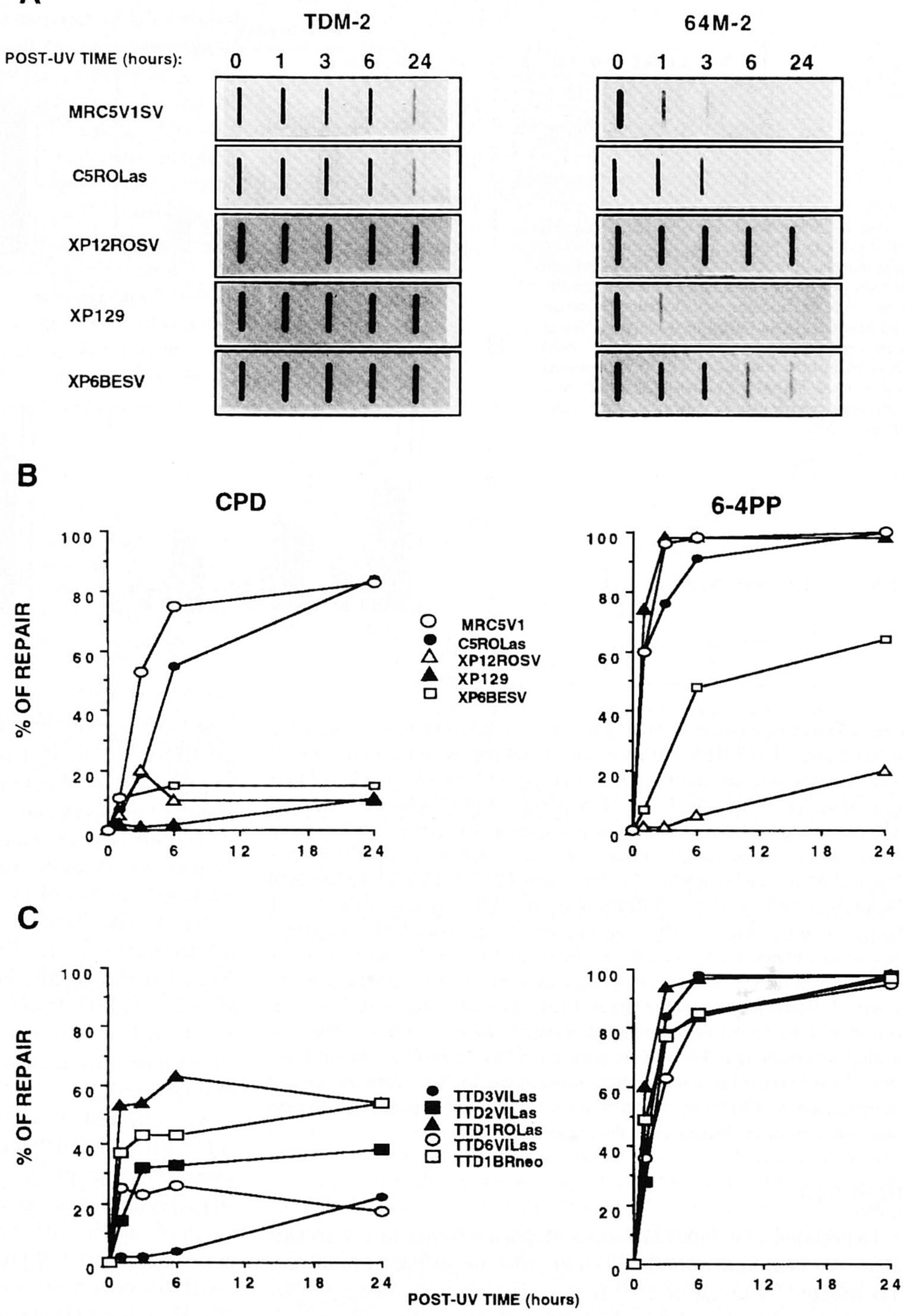

Fig. 3. Repair kinetics of CPD and 6-4PP lesions in SV40-transformed wild-type, XP (*A* and *B*) and TTD (*C*) cells. *A*, autoradiograms of immunoblot experiments carried out with genomic DNA samples of irradiated (15 J/m^2) cells for each indicated postirradiation time, and with anti-CPD TDM-2 or anti-6-4PP 64M-2 mAbs (*left* and *right*, respectively). *B*, percentage of repair is expressed as the residual intensity of bands for post-UV samples over time 0 samples, quantified by densitometric scanning of blots in *A*. *C*, repair kinetics of CPD and 6-4PP in TTD cells. Data points were averaged for three separate experiments, and the SE for each experiment was about ± 15% in these experiments, as well in those in Fig. 4.

Determination of Repair Kinetics of CPD and 6-4PP Photoproducts in Genomic DNA in Wild-Type XP and in TTD Cells. We examined whether specific removal of CPD and 6-4PP is an intrinsic property of TTD cells or only a different lesion processing involving foreign plasmid DNA molecules. Therefore, we carried out experiments by measuring the DNA repair level by using slot-blot analysis of CPD and 6-4PP photoproducts, probed with anti-CPD (TDM-2) and anti-6-4PP (64M-2) antibodies, in genomic DNA at different post-UV times. Fig. 3 shows the repair kinetics of CPD and 6-4PP measured in several SV40-transformed control cell lines (Fig. 3, *A* and *B*) and in TTD cells lines (Fig. 3*C*). In wild-type MRC5V1SV and C5ROLas cells, more than 90% of 6-4PPs are repaired within 6 h, and 80% of CPDs are repaired within 24 h, consistent with previously reported properties of wild-type cells (36). In XP-A cells, the extent of removal of both photoproducts does not exceed 20% for 6-4PP and 15% for CPD, indicating the severity of repair deficiency for both photoproducts, whereas XP129 cells (XP12ROSV revertant cells) showed normal repair of 6-4PP within 6 h and defective CPD repair, as described previously (37, 38). In XP6BESV cells, the CPD repair is extremely reduced (15%), and 6-4PP repair is 50% within 6 h, consistent with repair properties already shown for XP-D cells (21). Similar extent and kinetics of repair for both types of lesions have been found using other mAbs against CPD and 6-4PP (H3 and 1F7, respectively) for C5ROLas,

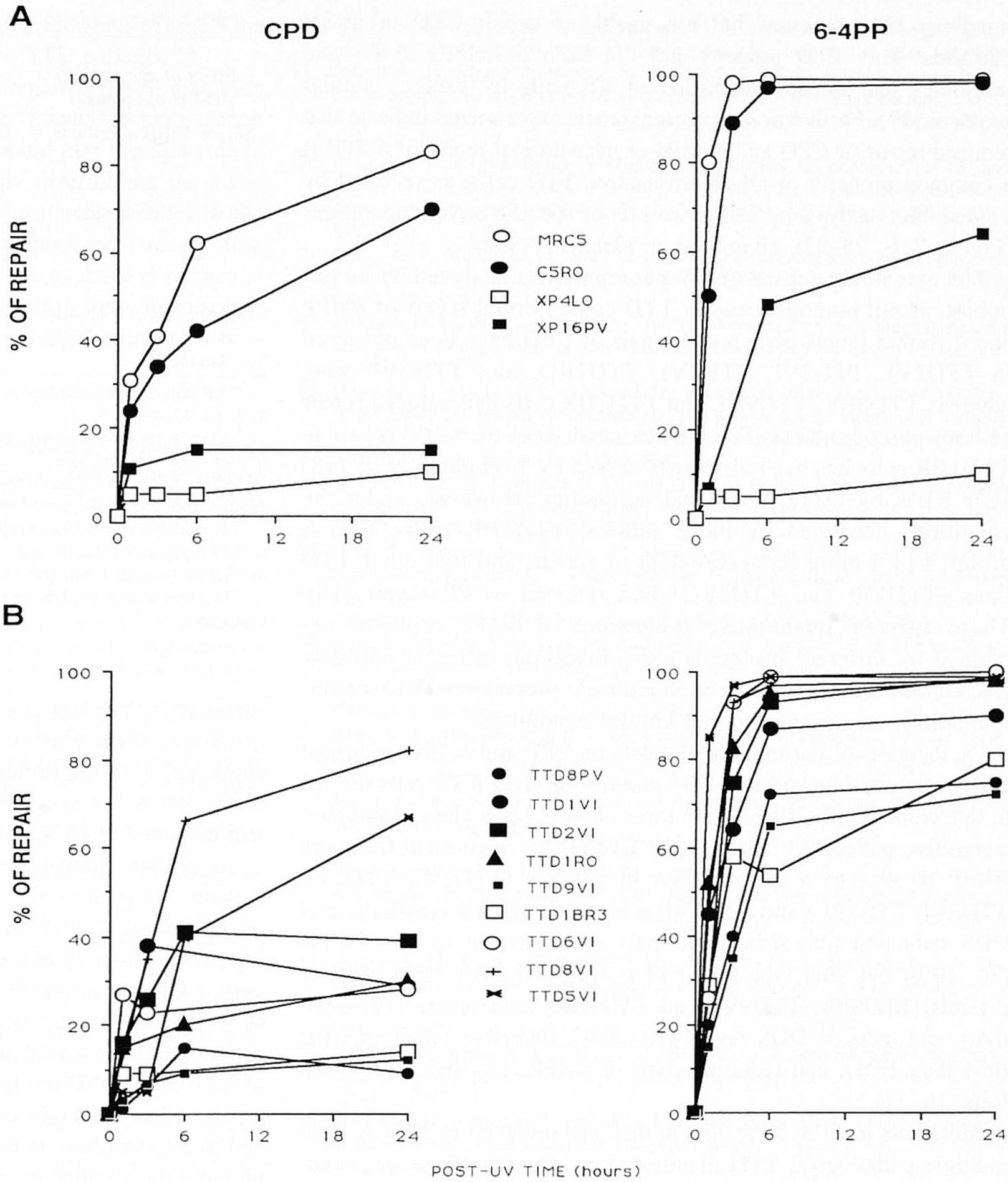

Fig. 4. Repair kinetics of CPD and 6-4PP in diploid fibroblasts from wild-type (MRC5 and C5RO) and XP (XP4LO and XP16PV) cells (*A*), and from photosensitive (TTD8PV, TTD1VI, TTD2VI, TTD1RO, TTD9VI, TTD1BR, and TTD6VI) and nonphotosensitive (TTD8VI and TTD5VI) TTD cells (*B*).

XP12ROSV, and XP129 (data not shown). TTD cells present a variable defect in the repair of CPD when measured 24 h postirradiation; between 20 and 60% of CPD lesions have disappeared, whereas repair of 6-4PP was essentially complete within 6 h at a rate of 80–100% of wild type for all TTD cells (Fig. 3*C*, compare with 3*B*). Similar results for both lesions have been found using other mAbs (H3 and 1F7) for TTD2VILas and TTD3VILas (data not shown).

To rule out that these repair properties in our TTD cells are influenced by SV40 transformation, we analyzed the kinetics of repair of CPD and 6-4PP in untransformed fibroblasts of TTD photosensitive individuals belonging to the three TTD genetic groups, and we compared these kinetics with those of wild-type, XP-A (XP4LO), and XP-D (XP16PV) cells, as shown in Fig. 4. The repair kinetics of CPD or 6-4PP in TTD cells from nonphotosensitive patients (TTD8VI and TTD5VI) are similar to wild type; 60–80% of repair of CPD was observed within 24 h, and more than 95% of repair of 6-4PP was observed within the first 6 h (Fig. 4*B*, compare to MRC5 and C5RO in Fig. 4*A*). Among TTD cells from photosensitive patients analyzed [TTD8PV, TTD9VI (TTD/XPD group), and TTD1BR3 (TTD-A group)] presented a very reduced extent of repair of CPD (less than 15%) and a moderately reduced repair of 6-4PP (50–70%). Cells from five other photosensitive TTD patients analyzed [TTD1VI, TTD2VI, TTD1RO, TTD3VI (not shown for TTD3VI; TTD/XP-D group), and TTD6VI, (TTD/XP-B group)] showed reduced repair of CPD (20–40%) but normal or almost normal extent of 6-4PP repair (85–100%; Fig. 4*B*). This indicates that the SV40 transformation does not significantly influence the repair properties of TTD cells studied here. Furthermore, a general feature is that 6-4PP repair seems to be relatively unaffected in TTD patients, whereas CPD removal is impaired.

DISCUSSION

In the present study we further characterized DNA repair properties of TTD cells with the aim of helping to explain the remarkable difference of skin cancer proneness between photosensitive patients affected by TTD or by XP. We provided evidence that, in all photosensitive TTD patients representing the three genetic groups thus far identified, the defective repair is mainly confined to CPD. By using a plasmid transfection procedure we demonstrated that *in vitro* removal of CPD by photolyase is sufficient to recover a normal expression level of a reporter gene in TTD cells. Because the reactivation of UV-irradiated plasmid has been proved to reflect the removal of UV photoproducts in a plasmid DNA transfected into human cells (39), our data demonstrate that TTD cells are able to repair 6-4PP and

nondimer photoproducts but are unable to repair CPD in pRSV plasmids. This TTD property has not been described so far and resembles that already identified in CS cells by using a similar protocol (17). Furthermore, genomic repair experiments indicate that reduced repair of CPD and normal or near-normal repair of 6-4PP is a common property of all photosensitive TTD cells, as revealed by immunoblot analysis by using two sets of specific mAb preparations (Fig. 1, Refs. 25–27), giving rise to identical results.

The extent of removal of UV photoproducts analyzed by immunoblots is not homogeneous in TTD cells; normal repair of 6-4PP and different levels of reduced repair of CPD have been observed in TTD1VI, TTD2VI, TTD3VI, TTD1RO, and TTD6VI cells, whereas TTD8PV, TTD9VI, and TTD1BR cells had reduced repair of both photoproducts (Fig. 4). Reduced level of 6-4PP repair in TTD1BR cells has been already observed by Broughton *et al.* (18) after RIAs by using polyclonal antibodies. However, under the conditions described by these authors, TTD1BR cells (TTD-A group) had a more reduced extent of repair, and two other TTD lines (TTD1VI and TTD1RO) had reduced 6-4PP repair (18). These different quantitative evaluations of 6-4PP could be explained by different protocols used, involving different antibody preparations (polyclonal or monoclonal), procedures (RIA or immunoblot), or, eventually, cell culture conditions.

On the basis of our immunoblot data on CPD and 6-4PP repair and previously described data on UDS and UV survival, TTD cells studied in this work may be shared into three classes: (*a*) a class of nonphotosensitive patients (TTD5VI and TTD8VI) having normal CPD and 6-4PP repair; (*b*) a second class of patients (TTD1VI, TTD2VI, TTD3VI, TTD1RO, and TTD6VI) with moderate UV sensitivity and UDS reduction (30–50% of normal) and defective repair of CPD (20–40%) but wild type repair of 6-4PP; and (*c*) a third class of patients (TTD8PV, TTD9VI, and TTD1BR) with severe UV sensitivity and reduced UDS levels (10–20%), defective repair of CPD (less than 15%), and reduced repair of 6-4PP (55–70%; Fig. 4 and Refs. 10, 11).[4]

Mutations in *XPB*, *XPD*, and in the gene involved in TTD-A yield multiple pathological TTD manifestations. Because these genes encode for proteins belonging to the TFIIH transcription complex (40, 41), alterations in one of these factors could also produce perturbations in gene expression and, thus, various clinical symptoms in patients, as we have already proposed (12), explaining the pathology of TTD. In photosensitive TTD patients, modifications in these three TFIIH proteins produce heterogeneous cellular responses to DNA damage: globally efficient NER of 6-4PP but low repair of CPD. Therefore, the molecular defect in TTD does not globally impair the potentiality of repair machinery. Mutations in these different factors could produce some subtle biochemical modification in TFIIH complex, giving rise to different interactions between this complex and damage recognition proteins, resulting in different processing of each UV photoproduct. One possibility is that for lesions for which recognition proteins have low affinity (such as CPD), the recognition protein has already dissociated from the injury before the mutated TFIIH complex has stably bound to it. Because of a reduced binding of the mutated TFIIH to the recognition complex, it might take too long for these complexes to find each other, or, alternatively, the stability of the association of complexes with each other and/or with the lesion would be reduced. This hypothesis could be further supported by the remarkable shape of the curve of removal of CPD in most photosensitive TTD cells (Figs. 3*C* and 4*B*). Initial rates are in fact indistinguishable from wild-type cells, but the CPD repair process seems to be blocked or exhausted, as if it is limited to a fraction of genome or if the repair capacity is exhausted at some points. This is consistent with the protein-DNA organization domains of the chromatin, where the accessibility of CDPs could be modulated by protein-mediated DNA structure in different chromatin clusters.

[4] Unpublished results for TTD9VI.

Table 2 *Putative effects of mutations in TFIIH components on clinical phenotype*

Effect of mutations in TFIIH components	Functions		Clinical phenotype
	NER	Transcription	
Severe TFIIH alterations	Defective	Normal	XP[a]
	Normal or defective	Defective	Lethal[b]
Subtle TFIIH alterations	Normal	Modulated	Non-photosensitive TTD[c]
	Modulated	Modulated	Photosensitive TTD[d]
Combination of "XP-like" and "TTD-like" TFIIH alterations	Defective	Modulated	Lethal[e]

[a] XP skin cancer phenotype is generally considered to be due to defective repair (Refs. 4, 7, 14, 42, 48).

[b] This is an obvious basic biological concept involving incompatibility of lack of transcription with cell life.

[c] Hypothesis of modulated transcription, envisaged in Ref. 12, involving the idea that subtle transcription alterations may cause TTD clinical symptoms.

[d] Hypothesis of modulated repair derived from our data on lesion-specific repair of the 6-4PPs repair in TTD cells, and showing wild-type repair (for the second class of patients) or slightly reduced repair (for the third class of patients).

[e] Our speculation explaining the absence of patients with double clinical XP and TTD symptoms.

To explain clinical features of TTD, including nonphotosensitive patients, we propose that different specific mutations in TFIIH components produce subtle molecular modifications of the complex yielding perturbations in transcription of specific genes involved in metabolic pathways responsible for typical TTD clinical symptoms, with or without defective processing of UV photoproducts. These modifications of TFIIH would not be compatible with the global NER defect of XP-B and XP-D groups. A double functional defect with impaired repair and subtle modification of TFIIH, yielding modulation in NER and/or transcription, may not be compatible with viability, thus explaining the absence of combined XP/TTD pathology (Table 2).

The current hypothesis on skin cancer development in XP is that a NER defect in nontranscribed DNA could be responsible for the accumulation of mutations, resulting in the expression of proto-oncogenes and inactivation of antioncogenes (7, 14, 42). TTD patients have quite different pathological features from XP patients, namely, no skin cancer and occasionally neurological disorders (7). TTD cells have proficient 6-4PP repair in the overall genome. Therefore, unrepaired 6-4PP in the overall genome might be considered a major lesion responsible for the accumulation of mutations, as it is the case for *Escherichia coli*, where mutagenesis at unrepaired 6-4PP sites is 9 times higher than at CPD sites (43, 44). However, high mutation frequencies were observed in pZ189 shuttle vector in TTD and CS cells, which, in the absence of CPD photoreactivation, are not significantly different from frequencies in XP cells (45, 46). Assuming that 100% of 6-4PP and other nondimer lesions are repaired in TTD and CS cells, mutations are due exclusively to unrepaired CPD. Because of the very low frequency of 6-4PP occurrence in plasmid target gene and the relatively high extent of 6-4PP repair in XP-D cells (about 50%; Fig. 3 and 4; see also Ref. 36), no difference in mutation frequency between XP-D and TTD/XP-D cells could be expected. Indeed, the striking contribution of 6-4PP on mutation occurrence in this shuttle vector has been assessed only after photolyase treatment of plasmid transfected into XP and CS cells; the mutation frequency was reduced to wild-type levels in CS cells, whereas it is still 100 times more elevated in XP-A and XP-D cells (46, 47). Further data from

experiments using a similar protocol would provide clues to assess the role of 6-4PP in mutation occurrence also in TTD patients.

Our results support the hypothesis that unrepaired 6-4PP may play the major role in the sequential molecular events in skin cancer development in XP patients. These lesions could play a major role in the initiation step of carcinogenesis, possibly determining the accumulation of mutations. On the other hand, proteins essential in the regulation of secondary subpathways, such as those for detoxification of UV-DNA damage and/or for modulating the expression of factors involved in cell cycle and/or cell immune control, may also explain the pathology without skin cancers in trichothiodystrophy.

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